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RESTORATION OF REGENERATIVE ABILITY IN LIGATED STEMS OF *TUBULARIA* IN AN ELECTRIC FIELD[1]

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There is evidence that the locus of regeneration and its direction may be controlled by the bioelectric field. Mathews (1903) measured potential differences along stems of hydroids and between hydranths and stems. The more apical (distal) regions were electronegative to more basal (proximal) regions at the surface. A gradient in potential, with the distal surface region more negative in most species, has been reported (Hyman, 1920; Hyman and Bellamy, 1922; Child and Hyman, 1926; Barth, 1934b). Two species, *Obelia commissuralis* (Lund, 1925) and *Eudendrium* sp. (Barth, 1934b) were found to be more positive distally.

Mathews (1903) suggested that the electrical polarity might determine the direction of differentiation. He also mentioned a preliminary experiment in which it appeared that an imposed current inhibited regeneration in one direction but not in the opposite direction. It was clearly demonstrated by Lund (1921) that an imposed direct current field can determine the polarity of regeneration in *Obelia*. Ordinarily pieces of stem of *Obelia* form hydranths at both ends. In fields of appropriate strength only that end, either distal or proximal, which faced the positive pole regenerated. Lund (1925) believed that regeneration was blocked at an end because the sign of the applied field was opposed to the sign of the charge across the coenosarc at that end. Barth (1934a) studied the effect of applied fields on *Tubularia crocea, Eudendrium* sp., and *Pennaria* sp. *Eudendrium*, like *Obelia commissuralis*, the species used by Lund, is positive at distal surfaces with respect to more proximal surface regions. *Tubularia crocea* and *Pennaria* on the other hand are more negative in their distal regions (Barth, 1934b). In all four of the forms the polarity of regeneration was reversed when the sign of the applied field was opposed to the sign inherent in the stems. This made it appear that the inherent field might determine morphogenetic polarity as does the applied field.

Marsh and Beams (1952) demonstrated that the morphogenetic polarity of pieces of planaria can also be reversed by an applied field. At low field strengths heads appeared at anterior ends only. In fields of intermediate strength heads formed at both ends. With stronger fields there was complete reversal of polarity and heads formed only at the original posterior ends. In planaria as in the hydroids it seemed that the electric field might determine the direction in which controlling information might pass from cell to cell. This as a possible mechanism was first suggested by Lund (1925).

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In the course of some grafting experiments in *Tubularia* it was learned that morphogenetic control is polarized (Rose, 1957). If graft and host were combined with the same polarity and the graft was in the distal position the graft suppressed the formation of like structures in the host. The host was still capable of making all regions more proximal than that being formed by the graft. When the graft and host were combined distal to distal with opposed polarity neither affected the other. Although tightly joined both formed distal structures. The repressive information appeared to move from distal to proximal only. The region of contact was like a watershed from which movement was away from the point of contact and not across it. The same relationship holds for *Stentor* (Tartar, 1964). An anterior region could prevent a potential anterior region from forming anterior structures if they were aligned with the same polarity. When combined anterior to anterior neither affected the other.

If, as some of the earlier investigators suspected, the polarity of differentiation were determined by the electrical polarity of the stem, one should be able to block the effect of graft on host by a field of appropriate strength and sign. It was learned that the effect of a graft on its host could be blocked by an applied field when the graft faced the negative pole (Rose, 1963).

Other experiments with *Tubularia* indicated that regional differentiation might be controlled by positively charged repressors moving in the bioelectric field (Rose, 1966, 1967ab). Positively charged regionally specific repressors were collected. These prevented differentiation of the level from which they were extracted and all more distal levels. For example, positively charged materials separated by electrophoresis from the middle region of the hydranth primordia prevented younger primordia from forming middle and distal structures but allowed all more proximal levels to develop. It appeared that differentiation proceeds because positively charged repressors from the most distal region are carried away from their locus of origin toward more negative proximal regions. The cells receiving the distal repressors do not form the most distal structures but do become the most distal structures from which they are not receiving repressors. The cells at the second level, as they differentiate, also release repressors which move proximally along the electrical gradient. Second level repressors prevent the formation of the second and first levels behind them but do not repress differentiation of the third level. As each level in its turn develops into the most distal structures not forming distal to it, differentiation proceeds until all levels have formed along the polarized electrical gradient.

If an artificial field of sufficient strength is applied to the combination of a distal graft attached to the distal end of a whole primordium the control of the graft is blocked when the combination faces the negative pole. In this case the applied field opposes the bioelectrical polarity and would tend to block the movement of positively charged molecules from graft to host (Rose, 1963).

If the theory of control by charged repressors is correct, one would expect a bioelectric field of a certain strength to be necessary for regeneration. Further, one should be able to weaken the bioelectric field and thereby prevent regeneration. The plan in the work to be described was to find a method of decreasing the normal bioelectric field to a level at which regeneration did not occur. After this was done stems were placed in a field of a strength already known to change

polarity in some cases. If they were then able to regenerate new structures after they had been given a new field, it would appear that a bioelectric field of a certain strength is necessary for regeneration in *Tubularia*.

Materials and Methods

The *Tubularia* most used in these studies was the common species found in the harbor at Woods Hole. This was formerly thought to be *Tubularia crocea* but is now known to fit the description for *Tubularia spectabilis* (Miller, 1969). *Tubularia spectabilis* from the Cape Cod Canal and *Tubularia crocea* from New Bedford were also used.

In all of this work it was important to know the original polarity of the regenerating stems. For this reason when stems were cut away from colonies, the distal end was cut away with a cut at right angles to the main axis of the stem. The proximal end was always cut obliquely. Whenever stems were ligated, distal and proximal ends and which way the stems faced in a field were indicated by coded colored ligatures.

When stems were subjected to an electric field they were held in place in grooves in slabs of 2 per cent agar in sea water. They were held in place by a covering layer of cotton organdie. This allowed pasteurized sea water dropping from capillary siphons to flow over and around the stems without dislodging them. The dripping directed to the region where stems were confined was at the rate of a liter in 6 to 8 hours.

The agar slabs were made and used in plastic trays measuring $17 \times 8.5 \times 0.8$ cm. The field was supplied by a Vokam power supply type 2541. The current density was maintained at 26 microamperes per square millimeter. The drop in potential was 0.3 to 0.4 volt per cm along the agar trays. The temperature was maintained at 14–15° C.

Measuring electrodes were silver-silver chloride mounted in glass tubes. Contact with the stems was by way of electrode tips of cotton umbilical tape impregnated with 2 per cent agar and moistened with sea water. Only electrodes whose inherent potential differences were less than 0.2 mv were used. Potential differences were measured with a Hewlett-Packard microvolt meter, model 425A. Stems to be measured were quickly dried on filter paper and placed across the cotton ends of the electrodes. Great care was exercised not to dry the perisarc too much. If this happened ends were injured, coenosarc shrank, air bubbles appeared and the injured regions became negative to nearby uninjured regions by approximately 4 mv. Such stems were discarded. It is much easier to measure the potential differences of ligated stems without injuring the stems. The ligatures act as purse strings closing the protective secreted perisarc over the living coenosarc.

Experimental Section

Potential differences were measured in 200 stems before and during transformation of distal ends to hydranths. It was confirmed that in general in *Tubularia* there is a graded potential difference with distal regions being more negative (Barth, 1934b). It was also confirmed that potential differences vary appreciably

and proximal regions may be more negative especially in the vicinity of a branch (Hyman, 1920).

Pieces of stem 10–15 mm long were removed from young colonies usually within two hours of the time they were collected and always within 24 hours. Both the colonies and the stems removed from them were kept in running sea water until they were measured in air as described in Materials and Methods. Potential differences were measured along the perisarc between the distal end and the middle and between the proximal end and the middle of the isolated stems. A typical experiment is recorded in Table I. At the left of the table are the readings in millivolts of the potential differences at the two ends with reference to the middle. In this experiment no measurements were made before 12 hours after the stems had been removed from the colony. In other experiments when stems were measured from 5 to 12 hours after isolation some had positive ends. This transient positivity had also been observed before but was usually over in from one to two hours (Barth, 1934b).

By 12 to 17 hours after isolation all of the distal ends had become negative by 0.9 to 1.5 mv. Most of the proximal ends had lower negative values at the twelfth and thirteenth hours, ranging from −0.3 to −1.0 mv. In the next four hours the highest negative value at a proximal end was 0.1 mv and the other four proximal ends had become positive. All of the stems formed hydranths at the distal end and none did at the proximal end. At the time of measurement three of the stems were showing the first signs of a primordium. The distal end and a region behind the future primordium had become more translucent. These three had readings of −1.2, −1.3 and −1.5 mv. The others were within a few hours of

TABLE I

Potential differences in millivolts between ends and middles in stems with open and with ligated ends

Hours	Open at both ends		Hours	Open at proximal end		Hours	Ligated at both ends		Hours	Open at distal end	
	D-M	P-M		D-M	P-M		D-M	P-M		D-M	P-M
12	−0.9	−0.6	12	−0.9	*−0.9*	12	−1.2	−1.2	12	−1.3	−0.7
12	−1.2	−1.0	12	−1.4	−0.8	12	−1.0	−0.4	13	−0.8	+0.3
12	−1.0	−0.8	12	−1.0	*−0.7*	12	−0.3	−0.1	13	−0.9	+0.6
13	−0.9	−0.8	13	−0.5	−0.3	13	−0.8	−0.2	14	−1.4	−0.4
13	−1.2	−0.3	13	+0.7	*−1.1*	13	+0.1	−0.4	14	−1.4	0.0
14	−1.5	+0.2	14	+0.1	*−1.2*	14	−0.4	+0.6	17	−1.5	+0.2
14	−1.5	+0.3	17	+0.6	*−0.9*	14	+0.1	+0.1	17	−1.6	+0.7
17	−1.3	+0.6	17	−0.5	−0.6	17	−0.3	+0.4	17	−1.7	+0.6
17	−1.3	−0.1	None regenerated at D under ligature. Five (those italicized) regenerated at P.			17	−0.3	−0.2	17	−1.2	0.0
17	−0.9	+0.2				None regenerated			All regenerated at D.		
All regenerated at D.											

Hours indicates number of hours since the stem was cut away from its hydranth. Measurements are in millivolts and were made between a point close to the distal end and a point in the middle (D-M). (P-M) indicates measurements made between a point close to the proximal end and a point in the middle.

reaching this stage. In this experiment and in all other experiments a relatively high negative value was found in regenerating regions in the period just before and during early primordium formation. Regions which were not going to regenerate developed lower negative potentials or positive ones.

In other experiments it was observed that regenerating ends became less negative or became positive during the late striate stage or when the base of the new hydranth was taking shape during what is called the pinched stage. This decrease in negativity with change to a positive charge had been observed by Barth (1934b).

A summary of typical potential differences between ends and middle are given for 3 stages in Figure 1. There is always high negativity prior to and during early regeneration at the distal end with a change of sign to a low positive reading for most by the time all levels of the primordium are delineated. The hours given for these stages are averages. Speed of regeneration varies considerably and striations first appeared as early as 17 hours and as late as 30 hours. It had already been observed by Barth (1934b) that when long stems were cut in thirds potential differences and speed of regeneration were greatest in distal thirds, less in middle thirds, still less in proximal thirds and the polarity was sometimes reversed in proximal thirds.

The figures given in Figure 1 are typical for rapidly regenerating stems. The figure given for the distal end of a typical 18 hour stem is −1.5 mv. The range for such preprimordium stems which subsequently completed regeneration was −0.9 to −1.7 mv. The non-regenerating proximal ends were less negative ranging from −0.8 mv to +0.6 mv. By the time striations had appeared at the distal end the proximal non-regenerating ends ranged from −0.4 to +0.4 mv. The distal ends where striations had appeared remained highly negative during the early striate stage.

Through the early stages of primordium formation up to the early striate stage high negativity in the range from −0.8 to −1.7 mv was found in regenerating

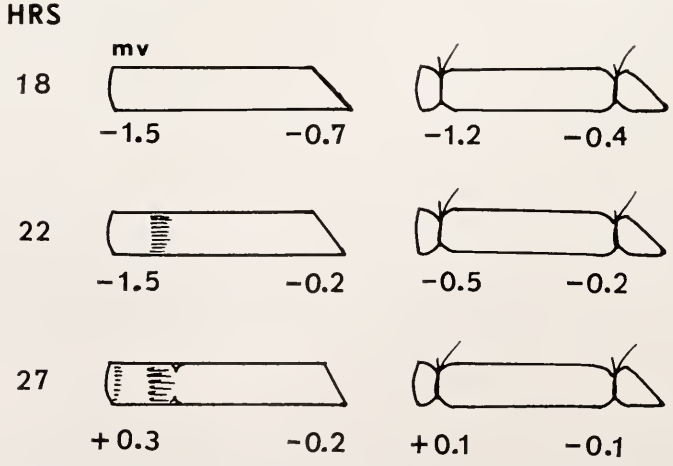

FIGURE 1. Potential differences in millivolts between ends and middle are compared in ligated and unligated stems at three different stages. The proximal ends are marked by an oblique cut.

ends. The dominated proximal ends showed values between −0.4 and +0.8 mv during the primordium stages.

In the cases covered in Table I and Figure 1 there was complete dominance of distal over proximal. When 10–15 mm pieces of stem were removed from colonies kept in the laboratory for several days or from colonies approaching dormancy in the harbor as the temperature approached 21° C, dominance was not complete. As a rule both ends transformed to hydranths. In these cases both ends became negative and remained so until the striate stage. The potential differences were lower in these more slowly regenerating stems showing decreased dominance. The range was from −0.5 to −1.0 mv. Other stems failed to regenerate. The highest negative value observed in them was −0.6 mv and the values ranged to +0.4 mv.

Ligatures at the ends of stems have an appreciable effect on potential differences measured along stems. They also prevent regeneration at the ligated ends. When stems were ligated at the distal end no regenerates appeared there. Five of eight stems released from the dominance of the distal end produced regenerates at the proximal end. At 12 hours three distal ends, even under their ligatures, had negative values of −0.9 to −1.4. These are in the range associated with incipient regeneration. Ligated distal ends failed to maintain high negativity and became less negative or became positive in the next few hours (Table 1). At the same time some higher negative values were found in the no longer dominated proximal regions. The 3 proximal regions which failed to regenerate had negative values of 0.3, 0.6 and 0.8 mv. The one which had been −0.7 mv at 12 hours regenerated very slowly. Regeneration in the five stems which finally regenerated at the proximal end required 20–30 hrs longer than for regeneration at open distal ends. Polarity was reversed in these stems but required a long time.

The high negativity at the distal ligated ends at 12 hours was in the range found in incipient regenerators. It was already known that ligatures do not prevent the early pre-primordial stages of regeneration. When stems were ligated at both ends for a part of the pre-primordial period and their distal ligatures removed, they regenerated as rapidly as sister stems which had not been ligated (Rose and Rose, 1941).

Even in the stems ligated at both ends (Table I) two at 12 hours had potential differences normally correlated with incipient regeneration. After 12 hours the values decreased below the level found in rapidly regenerating stems.

The potential differences observed between ends and middle were very much alike in stems with both ends open and in stems with the proximal end ligated. In both cases all distal ends regenerated and all proximal ends failed to regenerate (Table I).

The above results demonstrate that high negativity is associated with regeneration. The major question was whether the basic reason for failure to regenerate was failure to maintain the relatively strong electrical polarity. Since it was already known that an applied field could cause a reversal in morphogenetic polarity in *Tubularia* (Barth, 1934a; Levin, 1961), it seemed possible that ligated stems with too low potential differences might be aided to regenerate by giving them a field to replace that which they were losing while ligated.

There were 7 experiments in which some ligated stems subjected to an electric

field regenerated while ligated. Half faced the positive pole and half faced the negative pole. Seventeen of 79 with their distal ends facing the negative pole regenerated at their distal ends. None of 80 doubly ligated stems regenerated at their distal ends when they faced the positive pole. Four of these did regenerate at their former proximal ends which had faced the negative pole. In the 17 cases the applied field had acted in the same direction as the original inherent field. In the four cases in which regeneration had occurred at the proximal end the applied field had overcome the already weakened inherent field. Potential differences were measured along some of the ligated stems. The measurements were made in the period from 24 to 50 hours after the stems had been ligated. This was in the period before there was visible regeneration in most cases and when it was first observable in a few cases. Regeneration was considerably slower in ligated stems. Primordia did not appear until 48 to 60 hours.

It can be seen from Table II that 6 of the stems regenerated in which the distal end had faced the negative end of the field. Potential differences between distal ends and middle varied from −0.9 to −1.5 mv. Of the eleven measured stems treated in the same way, but which did not regenerate, the potential differences had been lower, ranging from −0.9 to +0.3 mv.

There were three of the measured stems which had faced in the other direction with their distal ends toward the positive pole which developed high negative potentials. These high readings were at the proximal ends toward the negative part of the applied field. Within 5 to 10 hours after the readings regenerates

TABLE II

The relationship between regenerative ability and negativity in stems ligated at both ends and subjected to a direct current field

Distal end faced negative pole				Distal end faced positive pole			
Regenerated at D(−)		Failed to regenerate		Regenerated at P(−)		Failed to regenerate	
D-M	P-M	D-M	P-M	D-M	P-M	D-M	P-M
−1.3	−0.9	−0.3	+0.3	+0.2	−1.6	−0.3	−0.3
−0.9	−0.5	−0.5	0.0	−0.1	−1.4	−0.1	−0.1
−0.9	+0.2	−0.4	+0.2	+1.2	−1.2	+0.5	+0.1
−1.1	+0.5	−0.5	−0.1			0.0	0.0
−1.0	+0.1	−0.6	0.0			−0.6	+0.9
−1.5	0.0	−0.8	+0.1			−0.7	+0.3
		+0.3	+0.4			−0.5	−0.1
		+0.2	+0.2			−0.2	−0.1
		−0.9	−0.1			−0.2	0.0
		−0.3	+0.3			−0.2	−0.3
		−0.7	+0.6			+0.5	+0.3
						+0.3	−0.4
						−0.7	−0.3
						−0.3	−1.1
						−0.5	−0.3
						−0.2	−0.7
						−0.8	−0.1

Readings in millivolts.

appeared at the proximal ends. This indicated that the applied field would not only maintain a field in the proper direction as in the former cases but that in these three cases the bioelectric field had been reversed. The regeneration was preceded by high negativity, 1.2 to 1.6 mv at the proximal ends.

Those doubly ligated stems which had faced the negative pole and did not regenerate had had a different range of potential differences, from −0.9 to +0.3 mv at their distal ends. The doubly ligated stems whose distal ends faced the positive pole ranged from −0.8 to +0.5 mv at the distal end and −0.7 to +0.9 mv at the proximal end.

The measuremetns were made in the period from a few minutes to 5 hours after the ligated stems had been removed from the imposed field. This means that in the successful regenerates the imposed field had caused a change in the bioelectric field which remained after the stems were removed from the imposed field.

In addition to the seven experiments in which some of the stems regenerated after being in or while still in the imposed field there were four other experiments in which no stems regenerated. Potential differences were measured in 32 of these stems. The highest negative value was −0.7 mv.

In all of the experiments a reading of −1.0 to −1.7 mv had predicted regeneration. A positive value or a negative value less than 0.7 mv had meant that no visible regeneration would follow. Values of −0.7 to −0.9 had no predictive value. Some regenerated and others failed. Potential differences are not steady and a stem with a reading of −0.7 to −0.9 mv could possibly have been slightly more or less negative than that most of the time.

In all of the experiments in which doubly ligated stems regenerated the stems had not been placed in the field immediately after ligation. After ligation they were held from one to 12 hours in a solution containing 10 mg of chloramphenicol per 100 ml of pasteurized sea water. The temperature was 15–16° C and the stems were not agitated. In general the longer, up to 12 hours, that the ligated stems were kept in this solution the greater was the effect of the subsequent treatment in the field.

A few stems were used from which small side branches had been removed flush with the main cylinder. Five such stems were ligated at both ends and the potential differences along the stem were measured shortly before a primordium could be expected to appear. The most negative region was the locus around the small opening in the perisarc. Just as Zwilling (1939) had learned after he had cut small openings in the perisarc of ligated stems, regenerates appeared in which all tentacles were aligned with their distal ends facing the small opening.

Discussion and Conclusions

In *Tubularia* any part of a stem can become a hydranth provided there is sufficient material. If there is too little to form a whole hydranth and just a part forms it is always a distal part. A small piece of totipotent tissue never forms anything but distal structures unless it lies behind a distal region. Then it forms the most distal structures not forming distal to it. The most distal region is the region from which organization spreads apparently by a series of repressions (Rose, 1957, 1967b, 1970).

The results of this work indicate that the most negative locus becomes the distal center of organization. Openings through the perisarc either at ends or along stems become negative centers. There is also an inherent electrical gradient with distal regions being more negative. If a distal region is ligated, a proximal end near the opening becomes the most negative. Imposed fields can also induce negative regions. Whatever the cause of the high negativity, the negative locus becomes a center of organization.

High negativity is necessary for regeneration. In ligated stems the negativity decreases to a level not found in regenerating stems. If the proper level of negativity is maintained in an artificial field, regeneration can proceed even under ligatures.

In addition the sign of an applied field can determine the sign of a bioelectric field and the sign of regeneration.

The measurements in this work were made at the external surface of the perisarc because of the relative ease with which this can be done. Lund (1925) had shown that the sign of the internal potentials is the reverse of that recorded at the surface. This means that internally where the repressors would be expected to move, the distalmost region is positive during the period when the repressors act. The internal bioelectric field would cause them to move from the positive distal region to the more negative proximal region.

It now seems clear that the bioelectric field is necessary for regeneration and not just a symptom of differences in chemical activity.

Summary

Electrical potential differences were measured in isolated stems of *Tubularia* during transformation of parts of the stems to hydranths. The transforming regions during the early stages of primordium formation were electronegative at the surface to adjacent non-transforming regions.

Potential differences lower than those measured in regenerating stems were obtained from non-regenerating ligated stems.

Ligated stems which were failing to regenerate because their potential differences were too low were given potential differences as great as those found in regenerating stems. When they were placed in an artificial field of appropriate strength, the ligated stems which regained the proper potential differences regenerated.

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